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13. ABSTRACT (Maximum 200 Words) The effects of ginseng on the prevention and treatment of breast cancer has not been studied. This research project was designed to examine the effects of American ginseng on breast cancer using well-established in vitro and in vivo experimental models. It is our hypothesis that ginseng, and its ginsenosides in particular, would inhibit the proliferation and growth of human breast cancer cells. Our results have shown that an extract of American ginseng inhibited MCF-7 and MDA-MB-231 cell proliferation in a dose-dependent manner. Furthermore, ginseng extract in the drinking water of female nude mice significantly decreased human breast cancer tumor growth. We have identified several different ginsenosides in ginseng extract and determined that only ginsenoside Rc potently inhibited breast cancer cell proliferation in vitro. These findings are the first to suggest that ginsenoside Rc may be responsible for the anti-proliferating actions of ginseng extract on human breast cancer cell proliferation. We have also shown that ginsenoside Rh2, a component of Asian ginseng, significantly inhibited breast cancer cell proliferation. More recent data suggested that ginsenoside Rc may inhibit breast cancer cell cycle progression, whereas ginsenoside Rh2, especially at higher doses, is cytotoxic. Thus, different ginsenosides may have very different mechanisms of action.				
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Introduction

The ingestion of Asian ginseng or ginseng root components has been reported to reduce the risk of human cancer and has been shown to decrease the rate of occurrence and proliferation of cancers in experimental animals. However, the effects of ginseng on the prevention and treatment of breast cancer has not been studied. This research project was designed to examine the effects of American ginseng (*Panax quinquefolium*) on breast cancer by studying the effects of novel ginseng preparations 1) on well-established human breast cancer cell models *in vitro*, 2) on human mammary tumor xenografts in nude mice *in vivo*, and 3) on carcinogen-induced mammary tumor development in female rats. Our hypothesis stated that specific ginseng components would inhibit the proliferation and growth of human breast cancer cells and would reduce the incidence of mammary tumor development in experimental animals. As breast cancer is one of the most prevalent and deadly diseases afflicting women today, a means of preventing and/or treating breast cancer through dietary supplements would have a significant impact on women's health.

Body

This is an annual report of Year 2 of the grant "American Ginseng in the Prevention and Treatment of Human Breast Cancer." In the Statement of Work,, two tasks were described which were initiated in Year 1, but were to be completed in Year 2. In Task 1, which involved testing the effects of American ginseng extract on human breast cancer tumor growth in nude mice and on mammary tumor formation in carcinogen-treated female rats, all sub-tasks have been completed. These tasks include 1) the treatment of nude mice with different concentrations of ginseng extract and inoculating them with MCF-7 cells, and 2) treating rats with various concentrations of ginseng extract and injecting them with the carcinogen N-nitroso-N-methylurea (NMU). In Task 2, which involved testing the effects of ginsenosides on MCF-7 and MDA-MB-231 cell proliferation *in vitro*, all sub-tasks have been completed. Much of the data described in Tasks 1 and 2 will be presented below. Task 3, which involves testing the effects of ginsenosides on human breast cancer tumor growth in nude mice and on mammary tumor formation in carcinogen-treated female rats, has been initiated and will be completed in Year 3. The specific tasks as described in this grant's Statement of Work include:

Task 1. Testing effects of American ginseng extract on human breast cancer tumor growth in nude mice and on mammary tumor formation in carcinogen-treated female rats.

- a. Prepare water extract of ginseng for HPLC analysis (month 1)
- b. Order mice and treat water with ginseng extract (months 1-6)
- c. Grow MCF-7 and MDA-MB-231 cells for inoculation (months 1-6)
- d. Inoculate mice and monitor tumor growth (months 1-8)
- e. Order rats and treat water with ginseng extract (months 7-12)
- f. Treat rats with NMU and monitor tumor formation (months 8-18)

Task 2. Testing effects of ginsenosides on MCF-7 and MDA-MB-231 cell proliferation *in vitro*.

- a. Grow and plate MCF-7 and MDA-MB-231 cells for inoculation (months 6-18)
- b. Treat cells with ginsenosides and measure proliferation (months 6-18)

Task 3. Testing effects of ginsenosides on human breast cancer tumor growth in nude mice and on mammary tumor formation in carcinogen-treated female rats.

- a. Order mice and treat water with ginsenoside combinations (months 19-28)
- b. Grow MCF-7 and MDA-MB-231 cells for inoculation (months 20-24)
- c. Inoculate mice and monitor tumor growth (months 21-28)
- d. Order rats and treat water with ginsenoside combinations (months 26-35)
- e. Treat rats with NMU and monitor tumor formation (months 27-35)
- f. Compile data for final analysis and presentation (months 34-36)

In Year 1, we demonstrated that treatment with 1% American ginseng extract in drinking water significantly decreased MDA-MB-231 tumor size in female nude mice. In a follow-up study, nude mice were started on 0.1 or 1% ginseng extract in drinking water and, 2 weeks later, inoculated with 5×10^6 MCF-7 cancer cells in a volume of 150 μ l into their right flank (Task 1b,c). Immediately following tumor cell inoculation, mice were implanted sc with a pellet containing 17 β -estradiol (0.72mg estradiol/pellet, 60-day release; Innovative Research of America, Sarasota, FL). Tumors were measured weekly beginning 11 days after inoculation. As shown in Figure 1, mice with 1% ginseng extract in their drinking water (n=12) exhibited a significant decrease in tumor size at 25 days post-inoculation when compared to water controls (n=12; $p < 0.05$). In the following 2 weeks, tumor size in ginseng-treated animals remained significantly decreased relative to controls ($p < 0.005$). The study was terminated at 39 days post-inoculation due to increased morbidity in the control mice. There were no differences in tumor size between animals on the 0.1% ginseng extract and water controls (data not shown). In Figure 2, representative control mice (upper two animals) and 1% ginseng-treated mice (two lower animals) indicate the presence of large necrotic tumors in the control mice relative to the smaller tumors in ginseng-treated mice. These studies support our previous findings which showed ginseng treatment decreased MCF-7 and MDA-MB-231 cell proliferation *in vitro*, as well MDA-MB-231 tumor growth *in vivo*, and suggest direct inhibitory effects of ginseng on tumor growth. In future studies, the nude mouse tumors would be examined to determine whether ginseng-induced apoptosis and/or inhibition of angiogenesis may be a mechanism by which ginseng and its ginsenosides inhibit tumor growth.

In a second study with ginseng extract (Task 1e,f), female Sprague-Dawley rats (n=19-20/treatment group) were given either plain drinking water (controls) or ginseng extract (0.1 or 1%) as their drinking water for 1 week prior to the administration of N-nitroso-N-methylurea (NMU; 50 mg/kg b.w.) via tail vein at 35 days of age. Our preliminary studies demonstrated that this experimental protocol produced NMU-induced

tumors in 75% of control animals when NMU was administered at 35 days of age (Table 1) vs only 20% of animals when administered at 80 and 87 days of age, as we had previously proposed. Ginseng was provided *ad libitum* in the drinking water for the duration of the experiment. The results show that when comparing control animals with ginseng-treated animals there were no differences in the percentage of animals in each treatment group that presented with mammary tumors (Table 1). Furthermore, there were no significant differences between treatment groups in number of days before tumor onset after NMU injection or the size of dissected tumors at approximately 3 months after NMU injection. There were 3 control animals with very large tumors (>1000 cu. mm) that were sacrificed before the end of the study and whose tumor size was included in the calculation of results presented in Table 1. Approximately 25% of tumor-bearing rats in each treatment group presented with 2 or more mammary tumors. The data shown in Table 1 represent the averages of all tumors detected. In conclusion, results indicate that ginseng treatment did not prevent tumor formation or affect the size of tumors in NMU-treated female rats when compared to non-ginseng-exposed rats. Although we had hypothesized that ginseng would prevent NMU-induced tumors and decrease tumor size, there may be several reasons why this was not realized. NMU is a powerful carcinogen that produces mutations in mammary tissue DNA that may not be able to be reversed or attenuated by ginseng exposure. In follow-up studies, ginseng effects on the onset of mammary tumors induced by treatment with other types of carcinogens, as well as on the mammary tumors that occur in normal aging female rats could be examined. The ability of ginseng to decrease tumor size in nude mice inoculated with human breast cancer cells, but not in rat mammary adenocarcinomas, could also suggest possible species specificity in the mammary cancer response to ginseng (ie, the therapeutic efficacy of ginseng could be influenced by species differences in ginseng pharmacokinetics). Lastly, the large quantity of powdered American ginseng root needed for this study was provided by Altex, Inc. (Kamloops, B.C.). This ginseng has not yet been analyzed by us for ginsenoside content, particularly for ginsenoside Rc, the ginsenoside which had previously been shown to be most effective in the inhibition of human breast cancer cell proliferation *in vitro*. Future studies could include a correlation of ginsenoside Rc content/concentration with the therapeutic efficacy of ginseng in its ability to inhibit cancer cell proliferation.

In Task 2a,b, the effects of ginsenosides, alone and in combination, on breast cancer cell proliferation were studied. We had previously shown that wide dose ranges of ginsenosides Rb1, Rb2, Rd, Re and Rf had no effects on MDA-MB-231 or MCF-7 cell proliferation. However, treatment with ginsenoside Rc produced significant dose-related decreases in cell proliferation, with a 75% decrease in cell number, relative to controls, after 8 days of treatment. More recently, a comparison of the effects of a wide dose range of ginsenoside Rh2 and Rc on MCF-7 cell proliferation were examined. Ginsenoside Rh2 is a unique ginsenoside component of Korean red ginseng and has recently been reported to inhibit breast cancer cell proliferation (Oh et al. 1999). MCF-7 cells were treated with ginsenosides Rh2 and Rc (1-50 μ M doses), alone and in combination, every 2 days for 6 days (Figure 3). Ginsenoside Rh2 produced significant decreases in cell proliferation with doses $\geq 15\mu$ M. The IC₅₀ for Rh2 was 18 μ M and at doses $\geq 40\mu$ M Rh2 killed 100% of the cells. Ginsenoside Rc significantly inhibited cell

proliferation with doses $\geq 10\mu\text{M}$ and the IC_{50} for Rc was $20\mu\text{M}$. After 6 days of treatment with Rc doses of $\geq 25\mu\text{M}$, cell number was decreased to approximately 35-40% of controls with no further decrease in cell number with higher doses. Combination doses of $18\mu\text{M}$ Rh with different doses of Rc ($1\text{-}25\mu\text{M}$) produced a more dramatic inhibition of cell proliferation than with either ginsenoside alone. Indeed, the IC_{50} of ginsenoside Rc when combined with $18\mu\text{M}$ Rh was $3\mu\text{M}$. A combination dose of Rh with $25\mu\text{M}$ Rc killed 100% of MCF-7 cells. None of the other ginsenosides (Rb1, Rb2, Rd, Re and Rf) affected the IC_{50} of ginsenoside Rc (data not shown). These results suggest that ginsenoside Rc may have cytostatic effects on breast cancer cell proliferation, whereas ginsenoside Rh may be cytotoxic. Indeed, in a follow-up preliminary study, MCF-7 or MDA-MB-231 cells were treated with either 1.0mg/ml ginseng extract or $50\mu\text{M}$ ginsenoside Rc for 72 hr, harvested, and subjected to cell cycle analysis using flow cytometry (Figure 4). Results indicated an increased percentage of MCF-7 and MDA-MB-231 cells in the G0/G1 phase and decreased percentage of cells in the S and G2/M phases of the cell cycle in ginseng and ginsenoside Rc-treated cells relative to vehicle controls. Future studies could more closely examine the mechanisms of action of ginsenosides Rc and Rh2 on the cell cycle and determine the effects of co-treatment with ginsenoside Rc and chemotherapy drugs currently used in the treatment of breast cancer on human breast cancer cell proliferation *in vivo* and *in vitro*. In Task 3 of this proposal, the effects of ginsenoside Rc on human breast cancer cell proliferation *in vivo* will be determined in nude mice.

Key Research Accomplishments (Year 2)

- A 1% American ginseng extract in drinking water decreased tumor size *in vivo* in female nude mice inoculated with MCF-7 human breast cancer cells.
- American ginseng extract did not affect NMU-induced tumor onset or tumor size in female Sprague-Dawley rats
- Ginsenosides Rc and Rh2 significantly decreased MCF-7 cell proliferation *in vitro*, and a combination dose of Rc and Rh2 was more potent in inhibiting cell proliferation than the effect of either ginsenoside alone.
- Whereas ginsenoside Rc appeared to have cytostatic effects on cell proliferation, ginsenoside was cytotoxic, suggesting different mechanisms of ginsenoside action on MCF-7 cells.

Reportable Outcomes (Year 2)

Abstract:

Murphy, L.L., Rice, J.A., Compardo, M.T. Ginsenoside Rc, a constituent of American ginseng extract, inhibits breast cancer cell proliferation *in vivo*. (*Submitted to 2001 AACR-NCI-EORTC International Conference, October 2001*)

Murphy, L.L., Rice, J.A., Zong, W. Ginsenosides Rc and Rh2 inhibit MCF-7 cell proliferation through distinctly different mechanisms. (*Submitted to American Society for Cell Biology Annual Meeting, December 2001*)

Invited Presentation:

Murphy, L.L. American ginseng and its effects on human breast and prostate cancers (Invited Oral Presentation at the Wisconsin Ginseng Growers Association in Wausau, WI in April, 2000)

Invited Presentation w/Abstract:

Murphy, L.L., Zong, W., Rice, J.A. Effects of American ginseng and Chinese herbal powder, alone and in combination, on human breast cancer MCF-7 cells *in vitro*. (New York 21st Century Chinese Medicine Forum, New York City, August, 2001) - this work was supported in part by American WildSenenergy Inc.

Manuscript:

Rice, J.A. and Murphy, L.L. Ginsenoside Rc, a constituent of American ginseng extract, inhibits breast cancer cell proliferation *in vivo*. (*In preparation for submission to Cancer Letters, 2001*)

Conclusions

The most notable findings of this research project include our results demonstrating that American ginseng extract in the drinking water of female nude mice decreases human breast cancer tumor growth, and that a component of this extract, ginsenoside Rc inhibits breast cancer cell proliferation *in vitro*. These findings are the first to suggest that

ginsenoside Rc may be responsible for the anti-proliferating actions of ginseng extract on human breast cancer cell proliferation, and that ginsenoside Rc itself should be further examined as a potential preventative or therapeutic agent for breast cancer. Preliminary studies indicate that ginsenoside may inhibit breast cancer cell cycle progression, whereas ginsenoside Rh2, a component of Asian ginseng, may be cytotoxic. Thus, different ginsenosides may have very different mechanisms of action. The inability of ginseng to prevent NMU-induced mammary tumor initiation and growth indicates a potential limitation to ginseng as a preventative against certain mutagenic carcinogens.

References

Oh, M., Choi, Y.H., Choi, S-H., Chung, H-Y., Kim, K-W., Kim, S.I., and Kim, N.D. Anti-proliferating effects of ginsenoside Rh2 on MCF-7 human breast cancer cells. International Journal of Oncology 14: 869-875, 1999.

Appendices

Please see Figures 1-4 and Table 1 on the following pages.

Effect of American Ginseng Extract in Estrogen-Treated Nude Mice Inoculated with MCF-7 cells

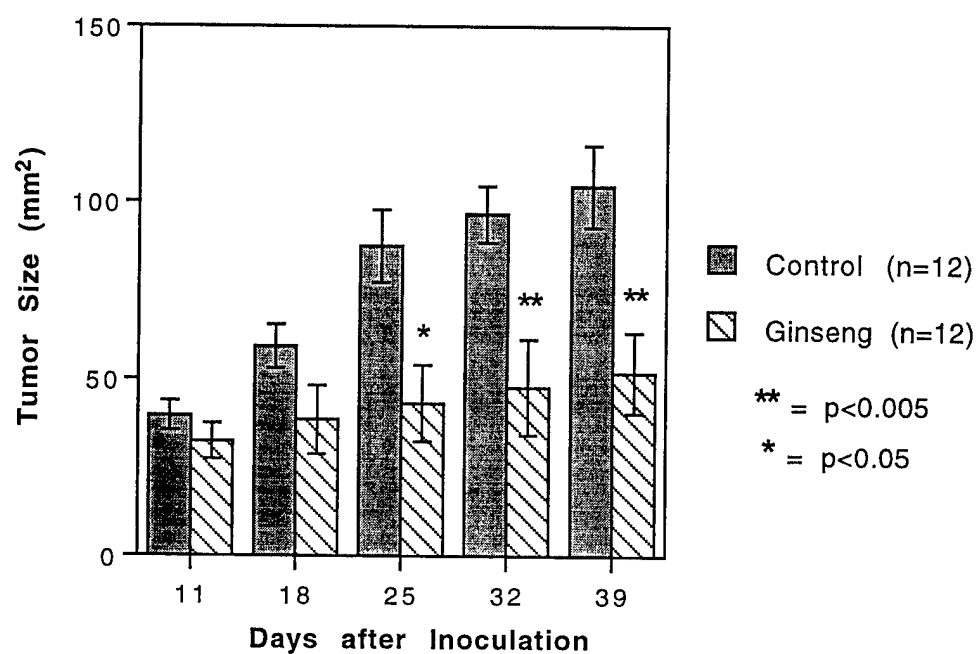


Figure 1 Effect of a 1% ginseng extract on tumor growth in female nude mice inoculated with MCF-7 breast cancer cells. Drinking water containing 1% ginseng extract was provided *ad libitum* to mice two weeks prior to inoculation with 5×10^6 MCF-7 cells. Mice were maintained on the 1% GE throughout the experimental period. Tumor size was measured weekly.



Figure 2 Representative nude mice from a control group (upper two mice) or 1% ginseng extract-treated group (bottom two mice). Note signs of necrosis in control mice. An estrogen pellet can be seen on the second mouse from the top.

Table 1
Effect of Ginseng Extract on Mammary Tumor
Growth in N-Nitroso-N-Methylurea (NMU)-Treated
Female Sprague-Dawley Rats

	No. of rats with tumors (percent)	Tumor onset (days)	Tumor size (cu. mm)
Control	15/20 (75%)	64.7 ± 7.0	669.9 ± ^a 235
0.1% Ginseng Extract	15/19 (79%)	60.9 ± 5.5	411.7 ± ^b 61
1% Ginseng Extract	16/20 (80%)	55.4 ± 4.0	359.1 ± ^b 79

All rats (n=59) were treated with NMU (50mg/kg b.w.) via tail vein at 35 days of age. Ginseng treatment (0.1 or 1% ginseng in drinking water) was initiated 1 week prior to NMU injection. Control rats received untreated drinking water.

Tumor onset was the number of days between NMU injection and detection of first tumor in each animal. Tumor size was determined as size of dissected tumor (cu. mm) on the day of sacrifice. ^a = average of 27 tumors. ^b = average of 26 tumors. Data are expressed as average ± SEM.

Effect of Ginsenosides Rc and Rh2, Alone and in Combination, on MCF-7 Cell Proliferation

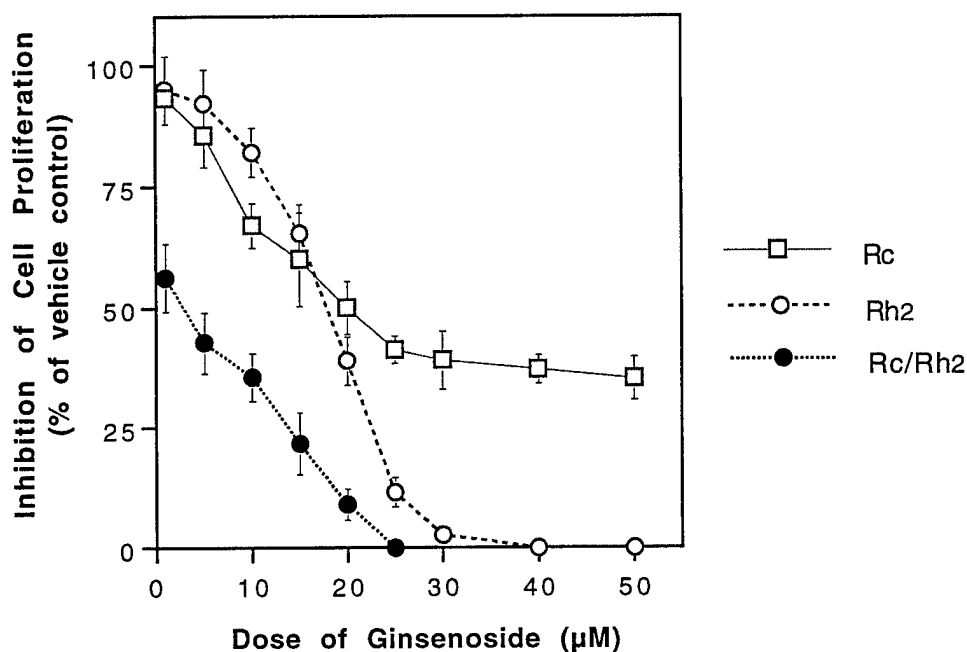


Figure 3 Effect of ginsenosides Rc and Rh2 on proliferation of MCF-7 breast cancer cells in culture. Cells were treated with a wide dose range of either ginsenoside Rc or Rh2, or a combination of Rh2 (18µM) and Rc, every 2 days. Cells were counted on Day 6 of treatment and data were graphed as a percent of vehicle control.

Effect of Ginseng Extract and Ginsenoside Rc on Cell Cycle Progression in Human Breast Cancer Cells

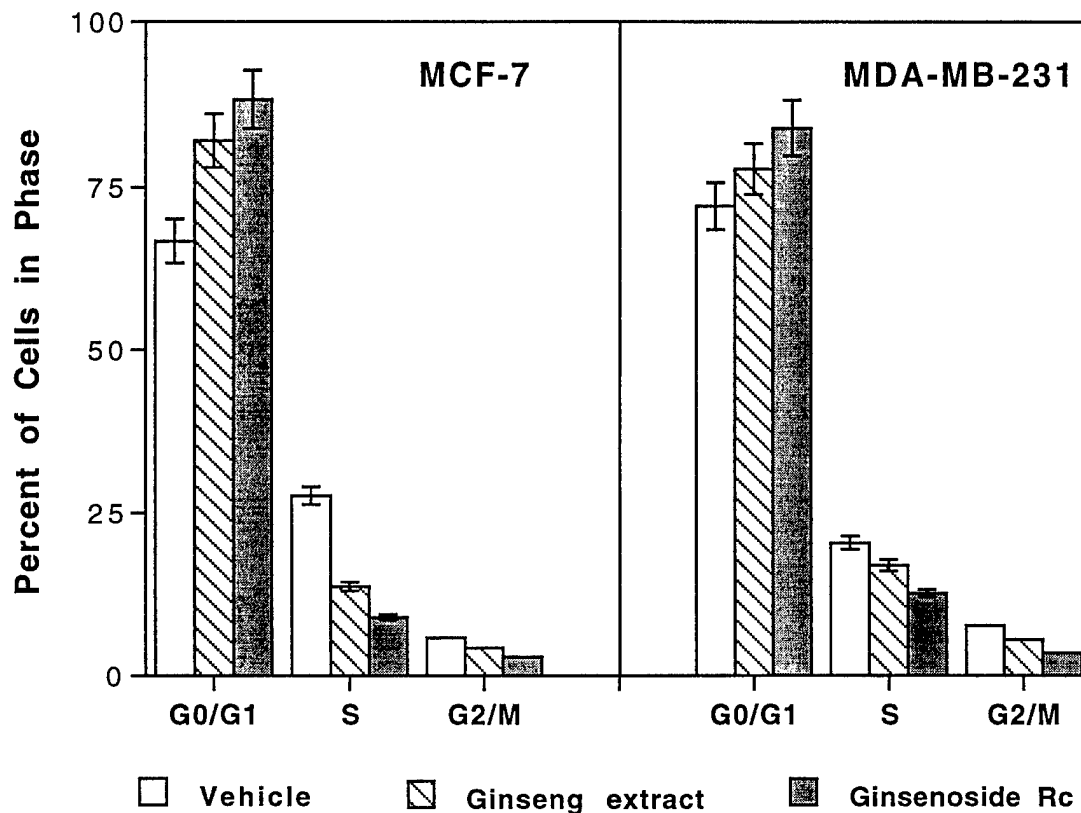


Figure 4 Effect of ginseng extract and ginsenoside Rc on cell cycle progression of MCF-7 and MDA-MB-231 breast cancer cells in culture. Cells were treated with either ginseng extract (1mg/ml) or ginsenoside Rc (50 μ M) for 72 hr. Cells were fixed and permeablized using an IntraPrep kit (Immunotech), washed with PBS and incubated with 20 μ g/ml Rnase A (Sigma) and 5 μ g/ml of propidium iodide (Sigma) for one hour at 4°C. The stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) for relative DNA content. Cell cycle histograms were obtained from 5 determinations, each with a total of 10,000 cells/group. The percentage of cells in the various cell cycle phases was determined using the Verity ModFit 2 software supplied by the manufacturer.